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Generation of Immortalized Murine Cell Lines with SV 40 T Antigen Derivatives Bearing Alterations in a Novel MHC Class II-Restricted Epitope

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Generation of Immortalized Murine Cell Lines with SV40 T Antigen Derivatives Bearing Alterations in a Novel MHC Class II-Restricted Epitope

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Running title: Generation of immortalized cell lines expressing altered T ag

ABSTRACT

The SV40 virus encodes an oncogenic large T antigen (T ag) protein that induces a strong immune response in mice. Four class I epitopes, I, II/III, IV, and V have been identified within the T ag, and epitope V has been shown to be immunorecessive. The four class I epitopes of T ag and the CD8 T lymphocyte response to them have been studied extensively, but little is known about CD4 T lymphocyte involvement in the immune response. A recent discovery by Mylin of a class II epitope within T ag will allow investigation of the role (s) of CD4+ T lymphocytes in the CD8+ T lymphocyte response to T ag and the cell-mediated control of SV40 T ag-induced tumors. A strategy to learn about the role(s) of CD4+ T lymphocytes includes immunizing mice with immortalized murine cells that express altered forms of T ag mutated in the class II-restricted epitope. CD4+ T lymphocytes will not identify and respond to T ag in the absence of the epitope they recognize; observation of the immune response in their absence will provide insight into their normal role(s). This project purposed to develop lines of immortal murine cells expressing mutated forms of the class II epitope in T ag for this purpose. The cells were produced by transfection with plasmids containing the desired mutations and the expression of T ag was verified using indirect immunofluorescence. Cell lines were obtained and preserved from cells transfected with all but one of the seven plasmids used. Positive IF results confirming T ag expression were found in all but two cell lines. The preserved cells will be used in future immunization studies to investigate the role of CD4 T lymphocytes and class II epitopes in the immune response.

INTRODUCTION

T lymphocytes, including both CD8+ and CD4+ T cells, are involved in cell mediated immunity. These T cells recognize antigens that are presented on Major Histocompatibility Complexes (MHCs) on the surface of host cells. T cells play a role in controlling cancerous tumors like those caused by the large tumor antigen (T ag) of the Simian virus 40 (SV40). The role of CD8+ in responding to SV40 T ag-induced tumors has been well-studied using animal models, but not much information is known about role of SV40 T ag-specific CD4+ cells.

T cells recognize foreign antigens that are displayed on the surface of host cells, become activated, and respond accordingly to facilitate cell mediated immunity. T cells are a positive control for the cellular immune response involving CD8+ killer cells and also regulate humoral immunity by controlling the functions of B cells, which produce antibodies specific to the foreign antigen (4, 5). Activated CD8+ T cells become cytolytic T lymphocytes (CTL), or killer cells, and lyse and destroy cells that present foreign antigens. CD4+ T cells, or helper T cells, respond to foreign antigens by producing cytokines that activate macrophages and B lymphocytes, which produce antibodies (1).

In order for T cells to be activated and respond to pathogens or viruses, they must come in contact with peptide antigens derived from the threat and identify these antigens as foreign. T lymphocytes are only capable of recognizing peptide antigens that are presented by MHC proteins on the surface of antigen presenting cells (APCs) (1). There are two types of MHC molecules. Class I MHC molecules bind to and present intracellular peptides that are present in the cytoplasm; these MHC-peptide complexes are recognized by CD8+ T lymphocytes, or CTL.

Class II MHC molecules bind to peptides derived from extracellular antigens that have been endocytosed and are degraded in intracellular vacuoles. Class II MHC molecules and the peptides they present are recognized by CD4+ T cells (1).

Epitopes are short peptides derived from an antigen that are presented on MHC molecules. A given antigen contains a limited number of epitopes that can be recognized by either CD4+ or CD8+ cells, and each epitope is recognized by either CD4+ or CD8+ T cells (10). Peptides of both self and foreign origin are able to bind to the broadly specific MHCs and be displayed, so it is the task of the T cells to determine which are foreign. One part of the peptide binds to a cleft in the MHC molecule and is held in place by anchor residues that fit into pockets in the MHC molecule, while other residues protrude from it to be available for T cell recognition (1).

Protein antigens that eventually are presented with class II MHC molecules are first bound and internalized by APCs and concentrated in membrane-bound endosomes where proteases degrade the proteins enzymatically into peptides which are able to bind to compatible class II MHC molecules (1). The class II MHC molecules are synthesized with the rough endoplasmic reticulum (ER). Nonpolymorphic proteins called invariant chains (Ii) associate with the MHC molecule, preventing epitope binding until it is directed to the endocytic compartments where the invariant chain is removed and epitopes are able to bind (4). Peptide binding stabilizes the complex, which is transported to the surface of the APC and presented for recognition by the CD4+ T cells (1). Class I MHC molecules are also synthesized in the ER and bind to peptides that have been transported there after being broken down in the cytosol (5).

T cells recognize foreign antigen-MHC complexes with T cell receptors (TCRs). Invariable regions of the receptors bind to the MHC molecules, and variable regions are available to bind to the antigens held by the MHC molecules (1). If both the MHC molecule and its peptide are bound, the peptide is recognized as foreign, an intracellular signaling process is initiated by membrane proteins, and the T cell is activated. The active T cell may produce cytokines and other effector molecules and divides rapidly, differentiating into memory cells and effector cells which lyse cells presenting the antigen (CD8+ T cells) or stimulate B cells, macrophages, and other cells (CD4+ T cells) (1).

The simian virus 40 (SV40) has been used extensively for the study of eukaryotic processes, including cell mediated immunity. SV40 is a polyoma DNA virus that has been shown to induce cancerous tumors in rodents (2). SV40 is highly immunogenic, causing both an antibody response to T ag and cytotoxic activity in the organisms that it infects. The replication protein and transforming protein encoded by SV40 is the Large T antigen (T ag). The T ag promotes continuous cell division by binding to and inactivating two tumor suppressing proteins, p53 and pRb (2). Their inactivation induces immortalization of the cell. The SV40 T ag contains four CTL epitopes. Epitopes I, II/III and V are H-2D^b-restricted, and epitope IV is H-2K^b-restricted. A hierarchy has been found among epitopes I, II/III, IV, and V; strong CTL responses are routinely detected against epitopes I, II/III and IV, but not against epitope V, which is immunorecessive (10).

The immune response to the CD8+ epitopes has been heavily studied, and CTL responses have been shown to be able to control tumor growth in mice (16). Not much is known about the importance or mechanism of the CD4+ response, although studies suggest it may play an important role (16). Unpublished studies by Mylin have located an epitope on the T ag that is recognized by CD4+ T cells in C57BL/6 mice (3,11). Further studies involving this epitope will investigate the role of CD4+ T cells in response to T ag, and may discover additional CD4+ specific epitopes on T ag. The proposed project will produce immortal cell lines expressing T ag in which the known CD4+ epitope has been mutated or removed, to be used in future immunization studies.

Materials and Methods

Kidney Cell Preparation

To obtain kidney cells for use in tissue culture, kidneys were harvested from male C57/BL6 mice. Each mouse was sacrificed by vertebral dislocation, and its back was wet with ethyl alcohol. A sterile scissors was used to make an incision through the skin down the center of the spine, and incisions were made on either side of the spine through the tissue underneath. The kidneys were located and removed using forceps and scissors and were placed in a 15 ml conical tube filled with 10 ml of Dulbecco's media (supplemented with 10% fetal calf serum, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 100 μg/ml kanomycin, 20 mM N-2-hydroxyethylpiperazone-N-2-ethanesulfonic acid (HEPES) and 15% (w/v) sodium bicarbonate). Unless otherwise indicated the media referred to is this media (10).

One at a time, each kidney was placed, with some media, into a 60 mm Petri dish. The kidney capsule was removed with sterile forceps and a scalpel was used to cut the kidney into pieces small enough to be easily taken up by a pipette. The pieces and media were transferred to a 15 ml conical tube, which was centrifuged for 5 minutes at 1000 rpm to pellet the cells and kidney chunks. The supernatant was aspirated with a vacuum and pipette, and the cells and chunks were re-suspended in 10 ml of 1% (w/v) Trypsin (10X trypsin in Trypsin diluent, pre-warmed to 37° C). The suspension was vortexed thoroughly for 1 minute, and placed in a Rollo drum to be continually rotated for 15 minutes. The chunks were allowed to settle after removal from the drum, and the supernatant was transferred to a 50 ml conical tube containing 20 ml of

media with 10% fetal calf serum. The tube was placed on ice. The chunks were re-suspended in Trypsin, vortexed, mixed, and supernatant was removed three more times. After the supernatant was removed the final time, the chunks were re-suspended in 15 ml media and allowed to settle; this supernatant was added to the 50 ml conical tube as well. The 50 ml conical tube containing the supernatant from all 4 repetitions with Trypsin was centrifuged for 5 minutes at 1000 rpm, and the supernatant was aspirated (10).

The pellets from both kidneys of each mouse were re-suspended together in 10% serum media for a total of 36 ml. The media, containing the cells from both kidneys, was placed into a labeled T150 flask and incubated at 37˚C with loose caps in 5% CO_2.

Culture Maintenance

The kidney cell cultures were maintained throughout the project by passing the cells to new flasks as they became confluent (10). Cells were dislodged from the flask and separated into single-cell suspensions using Trypsin (2 ml/ T25 flask, 4 ml/ T75 flask, and 6 ml/ T150 flask). They were then added in concentrations of 1:2, 1:3, or 1:6 along with fresh media to new flasks for a total volume of 6 ml in T25 flasks, 18 ml in T75 flasks, and 36 ml in T150 flasks. If cells were not passed, the media was changed twice weekly by aspiration the media with a vacuum pipette and adding fresh media with 10% calf serum in the volumes listed above using a pipette.

Transfection of Cultures with Plasmids Containing Altered SV40 Tag Genes

The cells from all cultures were dislodged from the flasks using trypsin and combined in 50 ml conical tubes. The tubes were centrifuged for 5 minutes at 1000 rpm, the supernatant was aspirated, and the pellets were re-suspended in a total of 20 ml. The concentration of cells in the suspension was counted using a hemocytometer, and the cells were plated into 16 new T75 flasks at a concentration of $1x10^5$ cells per flask in 15 ml of T10x3 media containing no antibiotics. The following day, the flasks were transfected with 7 plasmids by the Fugene 6 (Roche) method according to the recommendations of the supplier (14). The plasmids were generated using the Promega mutagenesis system and encoded T ag that had been altered in the class II-restricted epitope (12, 13). In each of 7 1.5 ml microfuge tubes, 800 µl of media containing 40µl Dulbecco's, 0.5µl HEPES, and 0.4 µl sodium bicarbonate, with no serum or antibiotics was added with a micropipette. To this, 20 µl Fugene 6 reagent was added directly to the media without touching the sides of the tube, and tube was mixed by tapping the bottom gently with a finger (14). Two µg of plasmid was added to each tube, which was mixed by tapping with a finger and allowed to incubate at room temperature for at least 15 minutes. An eighth microfuge tube received the same contents without any plasmid. Half of the contents of each microfuge tube was added to one of the T75 flasks that was plated the day before and contained 15 ml of T10x3 media. The flasks were labeled 1a and 1b through 8a and 8b, according to what plasmid they had received. Table 1 depicts which flasks received which plasmids. The flasks were incubated at 37 decrees C with vented caps.

Harvesting of Foci

The flasks transfected with plasmids were observed for the appearance of immortal foci, which presented as cloudy spots to the naked eye, and densely confluent cells under a microscope. When a flask contained numerous foci, they were harvested (10). Sterile 9 inch pipettes were prepared to pick the foci by drawing them out in a Bunsen burner using forceps. The pipette tips were heated until soft, and then pulled diagonally with a forceps so that a bent, thin section was present at the tip. The end was broken off to create a smaller opening, which was smoothed by heating it in the flame while air was forced through the pipette. In the hood, these pipettes were attached to a rubber bulb to create suction. Under a microscope placed in the hood, the pipette tips were used to scrape off and aspirate cells from the foci in each flask. For each focus, the media and cells that were removed were placed into one well of a 12 well tray that contained 4 ml of media containing 10% serum. A total of six foci were harvested from each flask, and the 12 well plates were labeled according to the flasks the foci had originated in. Flask 1a and 1b were grouped together on a plate, etc. After harvesting, the media in each flask was changed (10).

The wells in each 12 well plate were observed. When the cells in some of the wells in each plate became confluent, they were passed to flasks (10). A total of two wells from each half of a plate were passed, or two wells per transfected flask (1a, 2b, etc). To pass the cells, the well was aspirated using a vacuum pipette, and rinsed with room temperature PBS to remove residual

media. 1 ml of Trypsin was added to each well. It was observed under a microscope until the cells became dislodged from the bottom of the well; tapping gently with a finger helped to speed this process without spilling neighboring wells. When the cells were dislodged, 3 ml of media was added to the well, and the cells were suspended using a pipette. The cells and media were transferred to a T25 flask containing 2 ml of media, and were incubated at 37°C. The T25 flasks were labeled according to which transfected flask and well the cells had originated in (2A1, etc).

Indirect Immunofluorescence Preparation: Coverslip Plating and Fixing

Once the T25 flasks containing cells harvested from the 12 well plates became confluent, the cells were trypsinized with 2 ml of Trypsin into a single cell solution, and 4 ml of media was added for a final volume of 6 ml (10). For each 25 ml flask trypsinized, 2 60 mm tissue culture dishes were labeled (bottom and cover) with the flask's identification number (1A1, etc); one was labeled 1:2, the other 1:3. Five sterile coverslips were spread on the bottom of each, and each was filled with 7 ml of media. Two of the 6 ml of solution containing cells from the flask were placed into the 1:3 dish, and three in the 1:2 dish with a pipette. Media was dispersed with the pipette to ensure that the cells would grow on the coverslips. The remaining ml of cell-containing media was placed in a labeled T150 flask already containing 35 ml of media. The dishes were incubated at 37 degrees until the cells became confluent.

To fix the coverslips for immunofluorescence viewing, the media was aspirated and each dish was washed with 5-7 ml of cold phosphate buffered saline (PBS) three times. After the third wash was aspirated, each dish was filled with 7 ml of very cold 95% ethanol and put in the -80◦C freezer for 30 minutes. After removal from the freezer, the ethanol was aspirated thoroughly and the dishes were placed in a hood for several hours to dry (10,17). They were then stored in a refrigerator.

Cryopreservation

Once the cells in the T150 flasks described in the section above became confluent, the cells were frozen to preserve the cell lines for future use (10). The media was aspirated, and the cells were dislodged into a single cell suspension using 6 ml of Trypsin. Smacking the flask with a hand helped to dislodge the cells. 16 ml of media was added to the flask, and the cells were suspended in it. The cell-containing media was divided evenly between two pre-labeled 15 ml conical tubes, which were centrifuged for 5 minutes at 1000 rpm. The supernatant was aspirated, and each pellet was resuspended in 1 ml of freezing media and placed into a small conical tube to be frozen and stored at -80˚C.

Indirect Immunofluorescence

For each focus-derived cell line (1A1, for example), three fixed coverslips were removed from the 60 mm tissue culture dishes using forceps and placed cell-side-up in 35 mm tissue culture dishes prelabeled with the foci line and the letters A, B, and C. Coverslips that contained

B6/WT-19 (10) (express wild type T ag) cells and coverslips containing B6/SCL-7 cells (do not express T ag) were also used, as a control for the IF procedure. Using a forceps, each coverslip was re-moistened by dipping it in PBS and blotting it sideways on a paper towel before returning it to the dish cell-side up. Immediately, 130 µl of primary antibody was applied using a micropipette, and the coverslips were incubated for 30 minutes. For each focus-derived cell line, the dish marked A received PBS in place of an antibody, B received antibody 419 (6), which binds to the middle of the T ag protein, and C received antibody 901, which binds to the C terminal end of T ag (6). After 30 minutes, each dish was washed 3 times with 5 ml of PBS. The coverslips were not allowed to dry out between washes, and the last 5 ml of PBS remained in the dish until the secondary antibody was applied (17).

One at a time, the PBS from each dish was aspirated and the coverslip was transferred to the lid of the dish with forceps. Immediately, 135 µl of secondary antibody (FITC-conjugate secondary specific for the primary antibodies 419 and 901) was applied to the coverslip, covering the surface. The coverslip was then incubated for 30 minutes while being protected from light and desiccation. After 30 minutes, the coverslips were washed 3 times with PBS, avoiding drying out between washes. After the third application of PBS, the coverslip was rinsed in distilled water using a forceps, blotted sideways on a paper towel, and placed into a pre-labeled 12 well plate containing blotting paper. The coverslips were allowed to dry thoroughly, and then were glued to prelabeled slides. On each slide, coverslips A, B, and C were fastened in order. A drop of slide glue was applied, and the coverslip was placed cell-side-down on the drop. No pressure was applied to the coverslips; they were allowed to settle by gravity into the glue. The slides were allowed to dry before they were viewed under a UV microscope (10, 17).

Each slide was viewed under a UV microscope to determine if the cells were expressing T ag. If fluorescence was concentrated in the nuclei of cells, a value of +, ++, or +++ was recorded based on the brightness of the fluerescence (+++ as the most bright). If only background fluorescence was viewed, a value of – was recorded.

RESULTS

Generation of immortalized cell lines expressing T ag altered in the class II-restricted epitope

Two kidneys were removed from each of three male C57BL/6 mice, and cells were harvested and allowed to grow in tissue culture. The cells from all kidneys were combined and plated in 16 flasks. Two of the flasks were transfected with each of the plasmids listed in Table 1 and depicted in Figure 1. Two received no plasmid. The flasks were observed until foci began to appear. When significant numbers of foci were present and/or had reached an appropriate size, six were harvested from each flask; each was grown in a well of a 12 well plate. The two wells from each flask that grew most rapidly were passed to larger flasks. These cell lines were plated on coverslips and cultured until enough cells were present to freeze for future use to preserve the cell line. Therefore, for each plasmid used in transfection, four immortal cell lines were obtained. Two of each were known to be independently derived from the other two, since they were obtained from flasks that were transfected separately. For example, cells in 2A1 and 2A2 are known to be distinct from 2B1 and 2B2. The cell lines were preserved by freezing at -80°C (Table 2). The dates on which they were frozen indicate the relative growth rate of each line: more rapidly dividing cell lines were frozen at earlier dates.

Cells transfected with plasmid 3 grew at a much slower rate than those transfected with the other plasmids (excepting 7). Three foci from each flask were expanded for preservation and

characterized using IF, for a total of 6 cell lines obtained. Flasks 7a and 7b, transfected with plasmid 7, did not develop foci and later were discarded due to mold. A T25 flask transfected with plasmid 7 at higher densities developed a few foci which were picked and transferred to a 12 well plate, but the cells from the foci failed to proliferate, so no cell lines were produced from that plasmid.

Characterization of preserved cell lines using indirect immunofluorescence to confirm T ag expression

The coverslips from each cell line were fixed and stored until they could be observed with a UV microscope. For each cell line, one coverslip was stained using a primary antibody, 419, that binds to the middle of the T antigen, around amino acid 250. Another was stained with primary antibody 901, which binds to the T antigen near the C terminus of the T antigen. Another coverslip was stained using PBS instead of a primary antibody, to be used as a control. All coverslips were treated with secondary antibody. After staining, coverslips were glued to slides and viewed under a UV microscope. If fluorescence was concentrated in cell nuclei, the brightness of the nuclei was recorded as +, ++, or +++, with +++ representing the most bright of fluorescing nuclei. If only background fluorescence, or fluorescing of only cell parts other than the nuclei, was visualized, the brightness was recorded as "-". If T antigen was being expressed by the cell lines produced, it would be concentrated in the nucleus and bind to the primary antibodies during staining. The observation of fluorescent nuclei indicated that the cell line expressed T ag. Therefore, if the nuclei fluoresced, it indicated that T antigen was being

produced in the nuclei of cells. Therefore, immunofluorescence represented a method of verification that the immortal cell lines produced and preserved were in fact a result of T antigen transformation.

Indirect immunofluorescence (IF) was used to characterize the cell lines preserved and two control lines, B6/WT-19 and B6/SCL-7 (Table 3) (10). The WT-19 cells are known to express wild type T antigen, and served as a control to ensure that the staining worked correctly. Each time, these cells showed bright (+++) fluorescence for both primary antibodies and were negative when stained with only PBS, verifying the effectiveness of the stain. The B6-SCL-7 and B6/TBS cells are known to be negative for T antigen production, so they served as a control to ensure that false positives were not occurring. Each of these coverslips was negative with both primary antibodies and PBS, ruling out the possibility of false positives.

The cell lines produced all were negative with the PBS staining, as expected. All the slides also showed fluorescence of the nuclei with both primary antibodies with the exception of cell lines 3A1 and 3A3, which had negative results. Most of the slides showed bright fluorescence; only a few were recorded as + or ++. It was verified in these cell lines that T antigen is being produced in the nucleus, and the cell has been immortalized by the SV40 virus.

Discussion

Foci developed in most of the flasks transfected with the plasmids, as expected. The rate of development and the rate at which the cells divided once harvested were much faster in the flasks that received plasmid than the flask that did not, demonstrating the effectiveness of T antigen at transforiming and immortalizing cells. The differing rates of immortalization and growth among cells transfected with different plasmids is an important observation. The rate of focus development and growth was inversely correlated to the severity of mutation in the T ag in the plasmids used for transfected. Cells receiving forms of T ag that contained more alterations and more severe mutations were not immortalized as quickly and the cells did not divide as rapidly. This is reflective of the changes in the T ag protein produced, which results in altered ability to bind to proteins p53 and pRb. Highly altered T ags do not efficiently bind p53 or Rb and therefore, do not efficiently induce immortalization or transformation (9). For example, plasmid 2 resulted in the fastest growth. The cells transformed by it appeared to grow even faster than those transformed by the wild type T ag. This plasmid contained substitutions with alanine of three anchor residues in the class II epitope located at residues 529-543. This is in the region of the protein that binds to proteins p53 and pRb, so the changes may have increased the ability of T ag to bind to and inactivate these proteins. Cell lines transformed by plasmid 3 grew very slowly. This plasmid contained deletion of the core 9mer of the class II epitope. This is a more severe mutation, resulting in T ag that has a different sequence of residues and likely a different shape and structure than the wild type T ag. These changes appear to have decreased the ability of T ag to bind to and inactivate proteins p53 and pRb, decreasing the ability to immortalize cells. Cells transfected with pLM 364-1 (plasmid 7) did not appear to develop foci at rates higher than the cells receiving no plasmid. This was expected because pLM 364-1

contained multiple mutations that affected key transformation domains of the T ag. The mutations inactivated each of the four known H-2^b-restricted CTL epitopes (I, II/III, IV, V).

IF characterization verified the expression of T ag in most of the cell lines preserved. The positive results, viewed as fluorescent nuclei, indicate that T ag was being produced. This is important because it rules out the possibility of spontaneous immortalization. The negative IF results for cell lines 3A1 and 3A3 indicate that spontaneous immortalization, not T ag, was responsible for their immortality. They did not produce T ag and will not be helpful in future immunization experiments because they do not produce the protein that is desired. These two lines were also the slowest growing of the lines obtained from plasmid 3. This is an expected result, because the spontaneous process is not as effective as T ag at inducing immortalization and cell proliferation.

The cell lines preserved (excepting 3A1 and 3A3) will be useful in immunization studies in the future because they produce the mutated forms of T ag that are desired, to initiate a cellular immune response in mice without the help of CD4+ T cells. The cells will be used to immunize mice. The presence of T ag will initiate an immune response by the CD8+ T lymphocytes, because the epitopes they recognize, the class I epitopes, are intact in most of the plasmids. The mutations in the class II epitope will prevent activation of the CD4+ T lymphocytes, so their activity will not be included in the response. The substitution with alanine of the anchor residues will prevent presentation by MHC molecules. If the epitope is not presented, it can not be recognized. The substitutions of the non-anchor residues will prevent binding and recognition by the CD4+ T cells, also preventing a response. The deletion will

prevent both presentation and recognition to prevent CD4+ T cell response. The differences in the immune response when CD4+ T cells are not involved will provide insight into what they normally do. The differences in the CD8+ T cell response will help to indicate whether CD4+ T cell activity helps or hinders the CD8+ response. Plasmids 6 and 7 contain mutation in the class I epitopes. These mutations will prevent CD8+ response. Therefore, the activity and activation of only CD4+ T cells will occur and can be observed, providing more insight into their role(s).

It is also possible that the cell lines produced will lead to the identification of more class II epitopes. The class I epitope V was only identified when the other class I epitopes were mutated or removed. It is possible that an immunorecessive class II-restricted epitope will be located in a similar process.

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FIGURE LEGENDS

Figure 1. Mutations in plasmids used for transfection. Plasmid 1 contains the wild type SV40 T ag. Plasmid 2 contains substitution with alanine of 3 anchor residues within the class II-restricted epitope. Plasmid 3 contains deletion of the core 9mer within the class II-restricted epitope of T ag. Plasmids 4 and 5 contain substitutions with alanine of 3 different non-anchor residues of the T ag class II-restricted epitope. Plasmid 6 contains deletions of the class I-restricted epitopes I and II/III and substitution with alanine in epitope IV. Plasmid 7 contains inactivated class I epitopes (16).

Figure 2. Typical IF results. A) Typical strong positive result; bright fluorescence concentrated in the nuclei indicate expression of T ag. B) Typical weak positive result. Dim fluorescence concentrated in the nuclei indicate expression of small amounts of T ag. C) Typical negative result. Background fluorescence only indicates no T ag expression.

TABLE 1. Mutations in plasmids used for transfection of tissue cultures.

Flask	Plasmid[a]	Plasmid Mutations
1a,b	pLM 234-1	wild type T ag
2a,b	1-29 pLMB6W-1-29	substitute 3 of 4 MHC class II anchor residue codons with Alanine
3a,b	2B3 p413508-2-2B3	delete core 9mer (amino acids 531-539) of class II epitope
4a,b	3-39 pLMBN603-3-39	substitute 3 non-MHC anchor residue side chains (NH2 half) with Alanine
5a,b	4-22 pLMBN604-4-22	substitute 3 non-MHC anchor residue side chains (COOH half) with Alanine
6a,b	pLM 363-13	delete epitopes I, II/III, 3 A substitution in epitope IV
7a,b	pLM 364-1	inactivation of each CD8 epitope to prevent binding
8a,b	no plasmid	

[a]Each plasmid was used to transfect 2 separate flasks of primary kidney cells (a and b).

TABLE 2. Cell lines preserved.

Flask	Plasmid	Focus[a]	Date Preserved[b]	# Vials Preserved
1a	pLM234-1	1A2	11/18/2008	2
1a	pLM234-1	1A3	11/15/2008	2
1b	pLM234-1	1B3	11/18/2008	2
1b	pLM234-1	1B4	11/18/2008	2
2a	1-29 pLMB6W-1-29	2A1	11/12/2008	2
2a	1-29 pLMB6W-1-29	2A2	11/11/2008	2
2b	1-29 pLMB6W-1-29	2B1	11/3/2008	1
2b	1-29 pLMB6W-1-29	2B1	11/6/2008	1
2b	1-29 pLMB6W-1-29	2B2	11/10/2008	2
3a	2B2 p413508-2-2B3			
3a	2B2 p413508-2-2B3			
3b	2B2 p413508-2-2B3			
3b	2B2 p413508-2-2B3			
4a	3-39 pLMBN603-3-39	4A1	11/14/2008	2
4a	3-39 pLMBN603-3-39	4A2	11/11/2008	2
4b	3-39 pLMBN603-3-39	4B1	11/12/2008	2
4b	3-39 pLMBN603-3-39	4B2	11/11/2008	2
5a	4-22 pLMBN604-4-22	5A1	11/11/2008	2
5a	4-22 pLMBN604-4-22	5A2	11/11/2008	2
5b	4-22 pLMBN604-4-22	5B1	11/11/2008	2
5b	4-22 pLMBN604-4-22	5B2	11/11/2008	2
6a	pLM 363-13	6A1	11/12/2008	2
6a	pLM 363-13	6A2	11/13/2008	2
6b	pLM 363-13	6B1	11/13/2008	2
6b	pLM 363-13	6B2	11/13/2008	2

[a]The foci harvested from flasks transfected with each plasmid were named and the cell lines obtained from each retained the name of the foci.
[b]The dates indicate the rate of development of each focus and the rate of growth of the cells harvested from it.

TABLE 3. Results of IF visualization.

Flask	Plasmid	Focus[a]	IF Results[b] PBS	419	901
1a	pLM234-1	1A2	-	+ +	+ +
1a	pLM234-1	1A3	-	+ +	+++
1b	pLM234-1	1B3	-	+++	+++
1b	pLM234-1	1B4	-	+++	+++
2a	1-29 pLMB6W-1-29	2A1	-	+++	++
2a	1-29 pLMB6W-1-29	2A2	-	+++	++
2b	1-29 pLMB6W-1-29	2B1	-	+++	+++
2b	1-29 pLMB6W-1-29	2B2	-	+++	+++
3a	2B3 p413508-2-2B3	3A1	-	-	-
3a	2B3 p413508-2-2B3	3A2	-	++	++
3a	2B3 p413508-2-2B3	3A3	-	-	-
3b	2B3 p413508-2-2B3	3B1	-	++	++
3b	2B3 p413508-2-2B3	3B2	-	++	++
3b	2B3 p413508-2-2B3	3B3	-	++	++
4a	3-39 pLMBN603-3-39	4A1	-	++	++
4a	3-39 pLMBN603-3-39	4A2	-	+++	++
4b	3-39 pLMBN603-3-39	4B1	-	++	++
4b	3-39 pLMBN603-3-39	4B2	-	+++	+++
5a	4-22 pLMBN604-4-22	5A1	-	+++	+++
5a	4-22 pLMBN604-4-22	5A2	-	+	+++
5b	4-22 pLMBN604-4-22	5B1	-	++	++
5b	4-22 pLMBN604-4-22	5B2	-	++	+++
6a	pLM 363-13	6A1	-	+	++
6a	pLM 363-13	6A2	-	++	++
6b	pLM 363-13	6B1	-	+	++
6b	pLM 363-13	6B2	-	+	++
B6/TBS		.	-	-	-
B6/SCL-7		.	-	-	-
WT-19	.	.	-	+++	+++

[a]Cell lines preserved were named according to the focus from which each was obtained.
[b]Indirect immunofluorescence was used with each preserved cell line, using PBS (control) and primary monoclonal antibodies 419 and 901. Results are indicated with – (negative) or +

(positive). The number of + indicates the strength of fluorescence in the positive results; +++ indicates the strongest fluorescence.

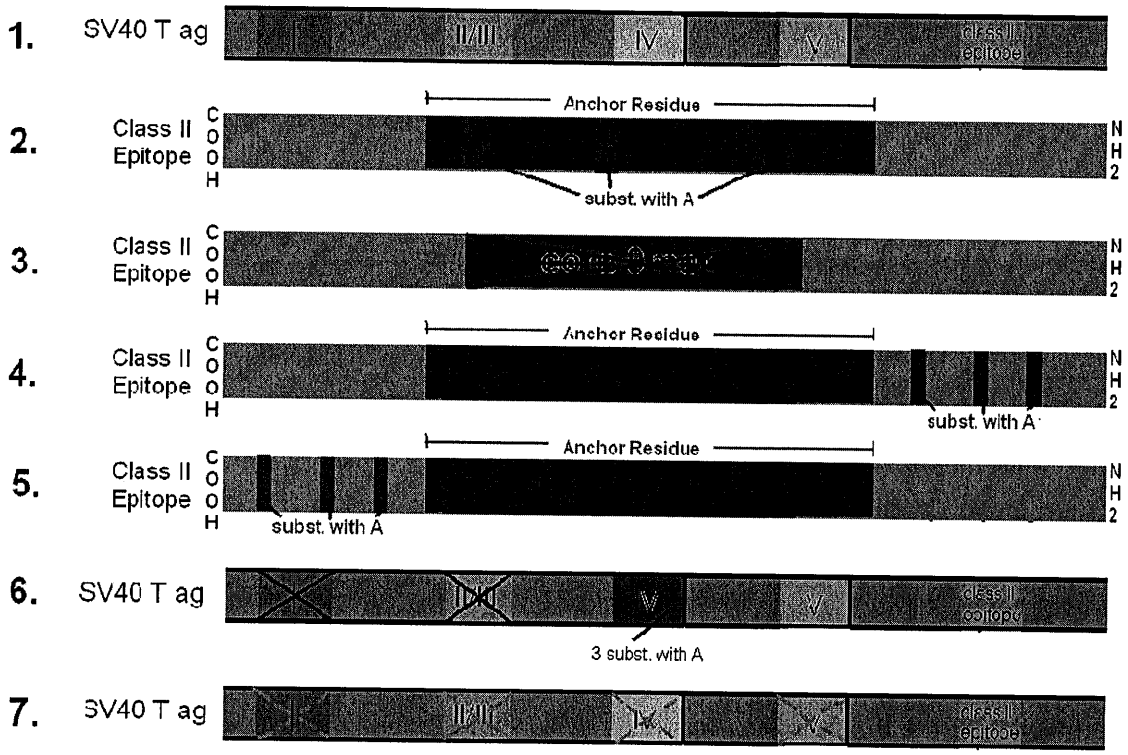
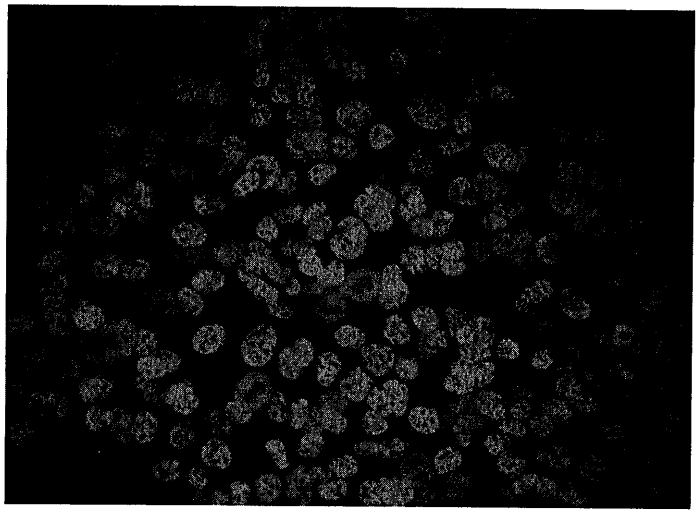
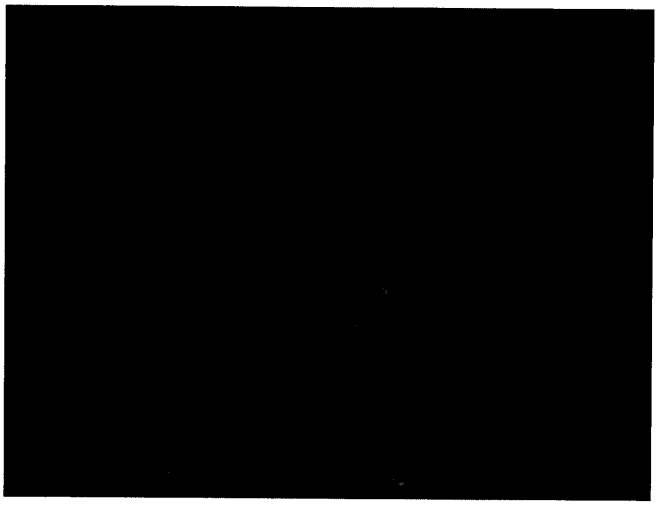


Figure 1.

A.



B.



C.

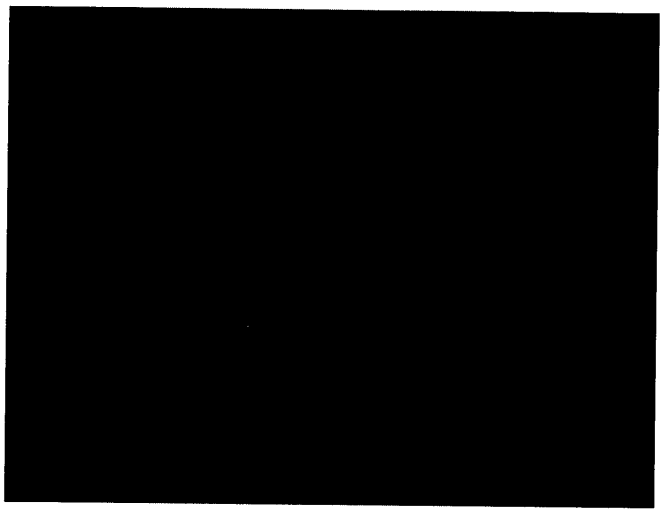


Figure 2.

Figure 2.